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Purification and characterization of *Streptococcus pneumoniae* palmitoylated pneumococcal surface adhesin A expressed in *Escherichia coli*

B.K. De[a,*], J.S. Sampson[b], E.W. Ades[b], R.C. Huebner[a], D.L. Jue[a], S.E. Johnson[a], M. Espina[a], A.R. Stinson[a], D.E. Briles[c], G.M. Carlone[a]

[a]*Division of Bacterial and Mycotic Diseases, and Biotechnology Core Facility Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA*

[b]*Pasteur Merieux Connaught Laboratories, Inc, Swiftwater, PA 18370, USA*

[c]*Department of Microbiology, University of Alabama, Birmingham, AL 35294, USA*

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Abstract

All *Streptococcus pneumoniae* isolates tested to date express a species-common lipoprotein designated as pneumococcal surface adhesin A (PsaA). This protein is cell-associated, hydrophobic, immunogenic, and genetically conserved. It is currently under investigation as a potential component in third-generation pneumococcal vaccine formulations. To overcome the problem of low-level expression of native hydrophobic PsaA in *S. pneumoniae*, and also of the recombinant PsaA (rPsaA) in *Escherichia coli*, we generated a stable *E. coli* construct expressing functional palmitoylated rPsaA (~10 mg/l of fermentation culture) using *Borrelia burgdorferi* outer surface protein A (OspA, a hydrophobic lipoprotein) signal peptide. By Western blot analysis, the chimeric rPsaA (~34 kDa) was detected in the cell lysate using anti-PsaA antibodies. It was partially purified by extracting the cell pellet with PBS/Triton X^R-114 buffers, followed by anion exchange filter chromatography. A trypsin digestion profile of rPsaA closely resembled that of the native protein, as revealed by SDS–PAGE/silver staining. Lipidation of rPsaA was confirmed by labeling recombinant *E. coli* cells with [^{3}H] palmitic acid and analyzing the labeled *E. coli* cells by Western blotting coupled with autoradiography. Further, analysis of purified rPsaA by mass spectrometry (MALDI-TOF) revealed a heterogenous spectrum with a major peak $(M+H)^{+1}$ of mass 33,384 Da (theoretical mass of palmitoylated rPsaA = 33,361 Da). Purified rPsaA was immunogenic in CBA/NCAHN-XID female mice following intranasal immunization with or without adjuvant, as determined by measurement of anti-PsaA serum IgG levels. These anti-PsaA antibodies reacted with both native and rPsaA polypeptides. Our data strongly suggest that *E. coli*-expressed rPsaA is palmitoylated and closely resembles the native protein in structure and immunogenicity. It was also observed to elicit measurable protection against nasopharyngeal carriage with *S. pneumoniae*. Published by Elsevier Science Ltd.

Keywords: Recombinant PsaA; Bacterial lipoproteins; *S. pneumoniae*

1. Introduction

Pneumococcal infections caused by *Streptococcus pneumoniae* are common worldwide [1,2], despite the availability of pneumococcal polysaccharide (PS) vaccine [3,4] and antibiotic therapy [5–8]. To date at least 90 different pneumococcal serotypes have been isolated, and the majority of them express many immunogenic proteins including pneumolysin [9], pneumococcal surface proteins A and C (PspA and PspC) [10–12], and pneumococcal surface adhesin A (PsaA) [13–16].

* Corresponding author. Centers for Disease Control and Prevention, MS G05, 1600 Clifton Road, NE, Atlanta, GA 30333, USA. Tel.: +1-404-639-3920; fax: +1-404-639-3115.

E-mail address: bkd1@cdc.gov (B.K. De).

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Acquisition of pneumococcal infection is generally from carriers rather than from infected individuals. In this regard, PspA has been shown to elicit protection against fatal bacteremia [17], whereas PsaA plays a significant role in protection against carriage [18]. It has been suggested that the best protection against pneumococcal infection is to induce herd immunity against pneumococcal carriage by incorporating a mixture of pneumococcal proteins, viz. PspA, PsaA or pneumolysin [18].

We have identified PsaA as a cell-membrane-associated lipoprotein expressed by all pneumococcal strains (>90 isolates) tested to date. It is a species-common, genetically conserved lipoprotein antigen which is encoded by the *psaA* gene having an open reading frame of 930 bp as a part of a polycistronic message. Recent studies from our laboratory have shown that immunization of mice with native PsaA [15] or insect cell expressed rPsaA [16] elicited anti-PsaA antibodies that protect mice against challenge with virulent strains of *S. pneumoniae*. We also generated five anti-PsaA monoclonal antibodies (mAbs) that reacted with all *S. pneumoniae* strains (>90 isolates) but showed no reactivity to 55 heterologous bacterial strains causing lower respiratory tract diseases [13,19]. Individuals with culture-confirmed pneumococcal disease develop anti-PsaA antibodies, which are detected by an enzyme-linked immunosorbent assay (ELISA) using purified native PsaA [20].

Efforts to prepare large quantities of native PsaA have been impeded by low-level expression by all pneumococcal strains and difficulty in purification [21]. Most cell-associated PsaA forms lipoprotein complexes (micelles) with the pneumococcal cell membrane, similar to other prokaryotic lipoproteins that bind strongly to the cell membrane [22–25]. Recently, X-ray crystallographic studies revealed PsaA as a metal (Zn^{2+}) binding membrane transport protein [26,27]. In this report, we describe simple methods that enable production of large quantities of purified authentic lipidated rPsaA. Our data strongly suggest that *E. coli* expressed rPsA is lipidated and closely resembles native protein in structure and immunogenicity.

2. Materials and methods

2.1. Materials

Carbenicillin, Triton X^R-114, Triton X^R-100, trypsin and isopropylthio-β-D-galactoside (IPTG) and lysozyme were purchased from Sigma (St. Louis, MO).

2.2. Bacterial strains and plasmid

Streptococcus pneumoniae type 6B [19] was grown and maintained as described previously [14]. PLN, a pneumolysin-negative virulent isolate of *S. pneumoniae* type 2/D39, was provided by Dr D. Briles (University of Alabama, Birmingham, AL). The pneumolysin structural gene is disrupted by an insert containing an erythromycin resistance gene. Competent *E. coli* DH5α cells (Life Technologies, Gaithersburg, MD) and HMS174-DE3/pLysS (Novagen, Madison, WI) were used for the generation of recombinant plasmid and expression of rPsaA polypeptides respectively. All recombinant *E. coli* strains were grown at 34°C in yeast extract-Trypton (YT) broth containing 0.8% NaCl and carbenicillin at 100 μg/ml as described [14]. The expression plasmid, pLF100 ($f1^+$, Amp^r, colE1, T7 based pET9 expression vector containing full length *B. burgdorferi* OspA sequence) was provided by Dr R.C. Huebner (Pasteur Merieux Connaught Laboratories, Swiftwater, PA).

2.3. Monoclonal and polyclonal antibodies

Anti-PsaA monoclonal antibodies (1B6, 1E7, 4E9, 6F6, and 8G12) and rabbit polyclonal anti-PsaA antibody used for the detection of recombinant PsaA polypeptides have been described elsewhere [19].

2.4. Construction of expression plasmid, pOPsaA.7

A DNA fragment (830 bp) encoding the functional *psaA* gene (i.e. without its signal sequence) was amplified by polymerase chain reaction (PCR) of *S. pneumoniae* type 6B genomic DNA as described [16]. The sense primer for PCR corresponded to the sequence (DE09: 5′-GGGCATGCGCTAGCGGAAAAAAAGAT) with two bases (GG) extension at the 5′ end along with a substitution '*C*' for 'T' (3rd nucleotide at 5′ end of mature *psaA* gene) to design a *Sph*I site (underlined). The reverse primer, complementary to the 3′ sequence, had a two-base (GG) extension (DE11: GGGGATCCTTATTTTGCCAATCCTTC) with a *Bam*HI site (underlined). To verify the sequence of the functional *psaA* gene (~843 bp with linkers), the amplicon was analyzed by restriction fragment length polymorphism (RFLP) with seven restriction endonucleases (*Bam*HI, *Hind*III, *Eco*RI, *Kpn*I, *Pst*I, *Sph*I and *Xho*I) [28]. The PCR DNA fragment was then digested with *Sph*I and *Bam*HI, purified by agarose gel electrophoresis, and inserted downstream of *Borrelia burgdorferi* outer surface protein A (*ospA*) signal sequence (51 bp) using an *E. coli* expression plasmid, pLF100, in subsequent steps [16].

The recombinant plasmid pLF100 expressing full-length *ospA* gene of *B. burgdorferi* under the control of a T7 promoter was selected to generate a chimeric *psaA* gene. In brief, pLF100 DNA was digested with *Bam*HI and *Sph*I, and the largest fragment (carrying *ospA* signal sequence only) was recovered by agarose

gel electrophoresis. This linear plasmid and *psaA* DNA fragment were ligated and used to transform the competent *E. coli* cells (DH5α). Recombinant clones were screened by Southern dot blot assay using a DIG-labeled oligonucleotide probe complementary to the junction between *ospA* signal sequence (underlined) and the 5′ terminus of *psaA* (DE10: GCCTTAATAG-CATGCGCTAGCGGAAAA). Several *E. coli* recombinant (pOPsaA.7) clones expressing *psaA* were verified by RFLP analysis of the plasmid DNA by using seven restriction enzymes as previously described [28]. *E. coli* competent cells (HMS174-DE3/pLysS) were transformed with a recombinant chimeric plasmid (pOPsaA.7) containing a functional *psaA* gene. To identify *E. coli* clones expressing PsaA polypeptide, several colonies were screened by immunoblot using an anti-PsaA monoclonal antibody (1E7) as previously described [16]. One stable recombinant clone (designated as HOPsaA.7) expressing chimeric *psaA* was selected for further studies.

2.5. Cycle sequencing of psaA

Authenticity of the recombinant clone (HOPsaA.7) expressing PsaA was determined by sequence analysis of the *psaA* gene with dye terminator cycle sequencing protocols (ABI PRISM dye terminators cycle sequencing kit, Applied Biosystems, Foster City, CA). In brief, recombinant plasmid DNA was isolated from HOPsaA.7 cells and used as a template for cycle sequencing using DE09 and DE11 primer pair for both strands, and the products were analyzed by a Model 373 Version 1.2 Sequencer (ABI) as described.

2.6. Isolation and purification of recombinant PsaA

To isolate cell-associated rPsaA, early log-phase cells (HOPsaA.7) were diluted to 1:10 (0.25 $OD_{600\ nm}$) with fresh YT broth (4 l) supplemented with carbenicillin (100 μg/ml) and 0.8% NaCl, and grown at 34°C to an $OD_{600\ nm}$ of 0.60. The cultures were induced with IPTG at a final concentration of 1 mM for 4 h and then harvested by centrifugation (10 min, 5000 *g* at 4°C). The cell pellets were resuspended in 20 vol of PEN buffer (50 mM phosphate, pH 8.0, 1 mM EDTA, and 50 mM NaCl) and treated as follows: (1) two cycles of quick freeze and thaw, (2) lysozyme treatment (1 mg/ml) in ice for 30 min, (3) DNase treatment (10 U/ml) at 25°C for 30 min, and (4) sonication twice for 15 s with setting 5 (Sonicator XL, Heat Systems, Farmingdale, NY). The cell homogenate was clarified by centrifugation (10,000 *g*) and the cell pellet was resuspended in 100 ml of PEN buffer containing 2% Triton X^R-114, extracted overnight at 4°C, and then clarified by centrifugation. This yielded a detergent soluble fraction (designated as DP1). The pellet was again extracted with another 100 ml of PEN/2%Triton X^R-114 buffer overnight at 4°C. The clear detergent solution (designated as DP2) was clarified by centrifugation. Both of these detergent solutions were incubated at 37°C for 25 min to allow for detergent phase separation and this was followed by centrifugation (10,000 *g*). The concentrated detergent phase (5–10 ml) was collected to a fresh tube, dissolved in 50 ml of cold phosphate buffered saline (PBS, 10 mM, pH 7.6), and dialyzed by using a Spectra/Por membrane of molecular weight cut-off 25,000 Da (Spectrum, Houston, TX) overnight against 10 mM phosphate buffer (pH 6.5) with several changes and was finally clarified by centrifugation to remove impurities.

To remove Triton X^R-114 from the isolated rPsaA polypeptides, an ion exchange filter chromatography was performed as previously described [16] with the following modifications. Briefly, the dialyzed detergent solution (DP1 or DP2; 10 ml, ~0.50 mg of protein/ml) was diluted with 10 vol of 10 mM Na-phosphate buffer (pH 7.6), adjusted with ethanol to a final concentration of 20%, and chromatographed through a strong anionic filter (Q100, Sartorius, Edgewood, NY) pre-equilibrated with washing buffer A (10 mM Na-phosphate, 0.1% Triton X^R-100, 20% ethanol, pH 7.6). Following two washes with 30 ml each of buffer A and then buffer B (10 mM Na-phosphate, 0.1% Triton X^R-100, 20% ethanol, pH 6.5), the filter was eluted with 24 ml of buffer C (100 mM Na-phosphate, 100 mM NaCl, 0.10% Triton X^R-100, 20% ethanol, pH 6.5). Two ml of fractions were collected and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 12%). Proteins were visualized by silver staining and confirmed by Western blot analysis using either rabbit polyclonal sera (1:5000 dilution) or mouse monoclonal anti-PsaA antibody (1E7, 1:1000 dilution) [19]. The micro BCA protein assay (Pierce, Rockford, IL), as well as the densitometric analysis (Bio Image, Ann Arbor, MI) of gels following SDS–PAGE, were used to determine the total protein content of these fractions using bovine serum albumin as standard.

To concentrate rPsaA eluted from Q100 ion exchange column, a desalting ultra-filtration with a low-speed centrifugation (3000 *g*) was performed using Centricon-100 microconcentrator (Millipore, Boston, MA). Briefly, a Centricon-100 filter was prewashed with buffer C; rPsaA solution was then passed through the filter by centrifugation (3000 *g*) for 90 min at 24°C. The retentate (200 μl) was washed twice with 2 ml of buffer D (10 mM Na-phosphate, 20% ethanol, pH 6.5) and clarified by centrifugation.

2.7. Removal of Triton X^R-100

To remove Triton X^R-100 from rPsaA preparation, 5 ml of Q100-purified rPsaA (~1 mg) was precipitated at −20°C with 2 vols of cold acetone overnight [29]. The precipitate was clarified by centrifugation. The pellet was washed twice with 2 ml of cold acetone, air dried and resuspended in 5 ml of buffer (10 mM phosphate, 20% ethanol, pH 6.5), and dialyzed extensively against the same buffer. This yielded rPsaA free from Triton X^R-100 which was used for various analytical studies, including mass spectrometry.

2.8. Trypsin digestion of rPsaA

To generate a trypsin digest profile of rPsaA, the acetone precipitated rPsaA was diluted (120 μg/ml) in trypsin diluent buffer (10% glycerol,125 mM Tris, 0.1% SDS, 1 mM $MgCl_2$, 0.005% bromophenol blue, pH 6.5) (protein fingerprinting kit, Promega, Madison, WI), and the solution was heated at 95°C for 5 min and then incubated with trypsin (final concentration 0.005%) for 30 min at 37°C. Native PsaA was also partially digested with trypsin under similar conditions. Partially digested peptides were analyzed by SDS–PAGE (15%) and visualized with silver staining [16].

2.9. [^{3}H]Palmitic acid labeling of rPsaA in vivo

The palmitoylation of rPsaA was assessed by labeling of early log-phase HOPsaA.7 cells with [^{3}H] palmitic acid (53.0 Ci/mol, Amersham, UK). Briefly, cells were grown in 10.0 ml of culture with or without IPTG (1 mM) in the presence of [^{3}H] palmitic acid at a final concentration of 10 μCi/ml [23]. Following incubation at 34°C overnight, cells were harvested, washed with cold PBS (10 mM, pH 7.2), and extracted with 2 ml of PEN/2% Triton X^R-114 buffer as described. The detergent phase extracts were separated by SDS–PAGE (12%) and transferred to a nitrocellulose membrane for Western blot analysis using an anti-PsaA mAb and then autoradiography, as described [23].

2.10. Mass spectrometry of rPsaA

The molecular mass of purified rPsaA was determined by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry [30]. Briefly, the detergent-free purified rPsaA (~1 μg) samples were mixed (1:9) with alpha-cyano-4-hydroxycinnamic acid (Hewlett-Packard, Palo Alto, CA), and 1 μl of the mixture was applied to the sample target. Mass spectral data were obtained from a REFLEX II MALDI-TOF mass spectrometer (Bruker-Daltonics, Billerica, MA). The accelerating voltage was set at 25 kV, using delayed extraction. The spectral data were calibrated by external standards.

2.11. Mouse immunization and intranasal challenge

Groups of 10 3- to 5-week-old CBA/NCAHNXID mice (Jackson Laboratories, Barr Harbor, ME) were immunized intranasally (i.n.) using purified rPsaA at the following doses: 150 ng or 500 ng per animal. For intranasal administration, 10 μl of each dose with or without 4 μg cholera toxin B subunit (CTB, List Biological, Campbell, CA) was prepared freshly with 0.85% physiological saline using a stock solution of 1 mg/ml of purified rPsaA. Mice were boosted twice with the same dose of rPsaA on day 7 and day 17 post-initial dose.

On day 14 following the final booster, saliva (~60 μl/mouse) and blood (100 μl) from the tail vein were collected from each mouse and analyzed for an IgG response by ELISA [21]. Six weeks after final boosting (day 38), mice were challenged with 10^6 colony forming units of PLN D39 suspended in 10 μl of 0.85% saline. On day 7 post-challenge, mice were euthanized and intranasal wash (6 drops) and blood (100 μl) were collected. Both blood and intranasal wash samples were serially diluted and plated to 5% sheep blood agar plate supplemented with 0.3 μg/ml erythromycin. Bacterial cultures were incubated at 37°C in a 5% CO_2 incubator, and colonies were counted following 24 h incubation.

3. Results

3.1. Expression of lipidated rPsaA

To facilitate the expression of recombinant PsaA, we constructed a plasmid, pOPsaA.7 with the functional *psaA* gene of *S. pneumoniae* type 6b, using an *E. coli*/T7 promoter expression vector. Sequencing analysis confirmed that chimeric pOPsaA.7 contained *psaA* sequence (data not shown) identical to the entire native *psaA* (870 bp) in frame with *ospA* signal sequence (51 bp) of *B. burgdorferi*. Our sequence data also predicted a theoretical molecular weight of 32,572 Da for the functional PsaA having 290 amino acids and an isoelectric point of 5.0 (data not shown).

HMS174 -DE3/pLysS cells were transformed with recombinant plasmid (pOPsaA.7), which generated a recombinant *E. coli* (HOPsaA.7) identified by immunoblot assay using an anti-PsaA mAb(1E7) (data not shown). Under reducing conditions, SDS–PAGE analysis of IPTG induced HOPsaA.7 cells revealed a major band composed of two closely related proteins with a molecular weight of ~30 to 34 kDa co-migrating with native PsaA (Fig. 1(A); lower arrow).

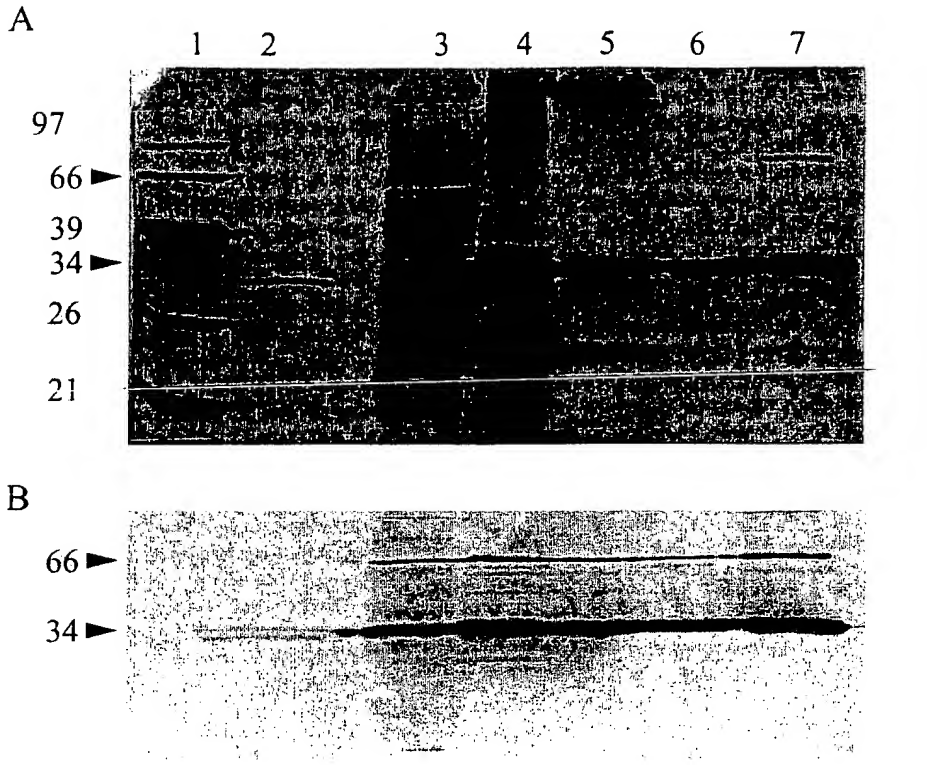

Fig. 1. Results of SDS–PAGE analysis of rPsaA by silver staining (A) and Western blot (B) using an anti-PsaA mAb. An average of 10 μg of total protein was electrophoretically separated in 12% polyacrylamide gels and silver stained (A). Lane 1, protein molecular markers; lane 2, purified native PsaA; lane 3, IPTG induced HOPsaA.7 cell lysate; lane 4, Triton X-114 extracted phase I (DP1); lane 5, Triton X-114 phase 2 (DP2); lane 6, Q100 purified rPsaA; lane 7, Centricon 100 enriched rPsaA. An anti-PsaA mAb (1E7) was used for Western blot (B) analysis. Low and high arrows indicate monomer and dimer forms of rPsaA polypeptide.

Western blot analysis using a mouse anti-PsaA mAb (1E7) confirmed that this band was PsaA (Fig. 1(B); lower arrow). Recombinant HOPsaA.7 cells also expressed a slowly migrating polypeptide with a molecular mass of ~65–66 kDa revealed by Western blot analysis (Fig. 1(A) and 1(B); upper arrow). No other detectable proteolytic PsaA fragments were found following IPTG induction of HOPsaA.7 cells. To investigate the hydrophobicity pattern of cell-associated rPsaA, both the aqueous phase and Triton X^R-114 buffer extracts of cell pellets were analyzed by SDS–PAGE and Western blotting using 1E7 mAb. The majority of cell-associated rPsaA polypeptides (>90%) were partitioned in the detergent phases (DP1 and DP2) [23], and were precipitated following phase separation at 37°C (Figs. 1(A) and 1(B); lanes 4 and 5). Thus, HOPsaA.7 cells predominantly expressed the membrane-associated rPsaA polypeptides, which mostly partitioned in the Triton X^R-114 phase and were designated as acylated rPsaA [22–25].

3.2. Purification of rPsaA

To remove Triton X^R-114 and host proteins from the purified rPsaA, an anion exchange filter (Q100) chromatography was performed. The majority of rPsaA was eluted from the strong basic filter Q100 column as a sharp peak (Fig. 1, lane 6) with a buffer (10 mM phosphate, 100 mM NaCl, 20% ethanol, 0.1% Triton X^R-100, pH 6.5). About 90% purity of rPsaA was achieved at a concentration of ~150–200 μg/ml, and the average yield was approximately 4–5 mg of purified protein per liter of fermentation culture. Although high levels of expression of rPsaA were noticed (~10 mg/l culture), the recovery of purified protein was about 50%. This could be attributed to the formation of rigid lipoprotein complexes with cellular lipid membrane, which allow only partial solubility of rPsaA in the presence of high concentration of Triton X^R-114 (2%). Further concentration of the purified rPsaA preparation was achieved by low-speed centrifugation using Centricon 100 filtration, which yielded rPsaA at a concentration of ~0.5 mg–1.0 mg/ml (Fig. 1, lane 7). This partially purified rPsaA appeared soluble only in the presence of nonionic detergents (Triton X^R-114 or Triton X^R-100) (data not shown). Following acetone precipitation of the purified rPsaA, it appeared soluble in detergent-free buffer (10 mM phosphate, 20% ethanol, pH 6.5) and suitable for various physicochemical studies.

3.3. Authenticity of lipidated rPsaA

The authenticity of rPsaA was confirmed by Western blot assays using a rabbit polyclonal antibody (Rab) and five mAbs raised against native PsaA [19]. All anti-PsaA antibodies detected the rPsaA polypeptide with a molecular mass of ~34 kDa (Fig. 2, lower arrow) as well as a slowly migrating recombinant protein with a molecular mass of ~66 kDa (Fig. 2, upper arrow). Furthermore, all these anti-PsaA antibodies reacted also with rPsaA polypeptides under non-denatured conditions, as revealed by immunoblot assays (data not shown). To determine the relatedness of native and recombinant PsaA polypeptides, both proteins were precipitated with acetone and partially digested with trypsin. The products were analyzed by SDS–PAGE (15%) and visualized by silver staining. A characteristic fingerprint pattern of four major fragments (Fig. 3) with molecular masses ranging from 30 kDa to 10 kDa, which co-migrated with those of native PsaA, were observed. Western blot analysis of the trypsin digested fragments of both native and rPsaA polypeptide demonstrated no reactivity with a rabbit anti-PsaA antibody (data not shown). Interestingly, a second ~32 kDa recombinant protein was detected using 15% SDS–PAGE (Fig. 3, lanes ± rPsaA-trypsin). This recombinant polypeptide was a product of the partial post-translational modifications [31] of rPsaA as revealed previously (Fig. 1, lower arrow). Furthermore, a computational amino acid analysis of PsaA (PROTEAN program, DNASTAR, Madison, WI) predicted at least 38 fragments generated by complete trypsin digestion (data not shown).

To identify the lipid moiety of rPsaA expressed in HOPsaA.7, cells were labeled with [^{3}H]-palmitic acid with or without IPTG induction. The most prominent labeled polypeptide appeared to have a molecular mass of ~34 kDa (Fig. 4(A), lower arrow), and its identity as PsaA was confirmed by Western blot analysis of the same membrane with an anti-PsaA mAb (1E7) (Fig. 4(B)). Interestingly, a slowly migrating rPsaA (~66 kDa) was detected by Western blot assay (Fig. 4(B), upper arrow), but it did not co-migrate with any [^{3}H] labeled polypeptide. Similar observations were noticed when cells were allowed to grow in the absence of IPTG. Thus both recombinant polypeptides partitioned mostly into the Triton X^R-114 phase as they were hydrophobic in nature, but only rPsaA with a molecular mass of 34 kDa was palmitoylated during postranslational modifications.

To determine the molecular weight of rPsaA (Triton X^R-100 free), it was analyzed by MALDI-TOF mass spectrometry. A heterogenous spectrum (Fig. 5) was observed with a major peak $(M+H)^{+1}$ of mass 33,384 Da and an apparently double charge species $(M+2H)^{+2}$ at m/z of 16,624 Da. Although these values appear slightly higher (< 1%) than that of the theoretical calculated mass (33,361 Da based on rPsaA without leader peptide), the method confirmed palmitoylation of rPsaA according to the proposed model for the prokaryotic lipoproteins [29–34]. Further analysis revealed a set of two comparatively weak peaks, $(M+H)^{+1}$ with a measured mass of 35,322 Da and a second peak $(M+2H)^{+2}$ with a molecular mass of 17, 692 Da. Interestingly, no peak corresponding to ~66 kDa protein was found under present experimental conditions. This data suggest that the palmitoylation of rPsaA by a heterologous signal peptide is possible.

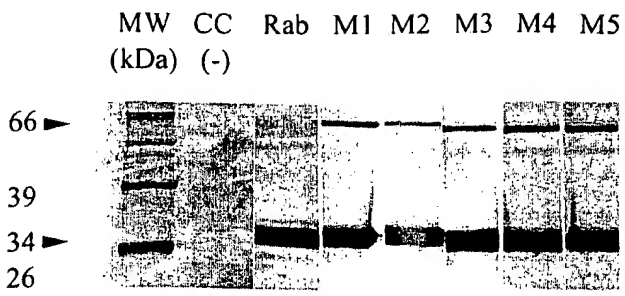

Fig. 2. Western blot analysis of rPsaA by anti-PsaA antibodies. Q100 purified rPsaA (~5 μg) was separated by 12% polyacrylamide gels, transferred to nitrocellulose membrane and analyzed by anti-PsaA monoclonal antibodies. Lane MW, protein standards; lane CC, casein control, lane Rab, rabbit polyclonal anti-PsaA antibody; Lanes M1 to M5 (1B6, 1E7, 4E9, 6F6, and 8G12), five anti-PsaA mAbs. Arrows indicate rPsaA polypeptides.

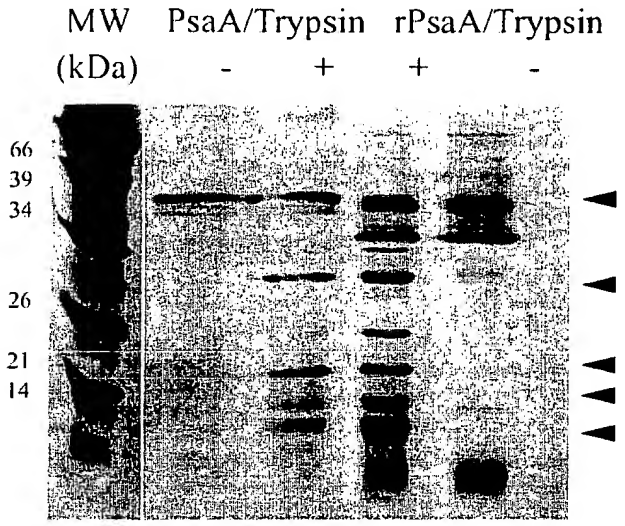

Fig. 3. Silver staining of native and rPsaA polypeptides generated by partial digestion with trypsin. Approximately, 1 μg of detergent-free native and rPsaA polypeptides were partially digested with trypsin. The products were separated by 15% polyacrylamide gels and visualized by silver staining. Lane MW, low molecular protein markers; lanes PsaA, native protein without and with trypsin; lanes rPsaA, recombinant protein without and with trypsin, respectively. Common fragments are indicated by arrows.

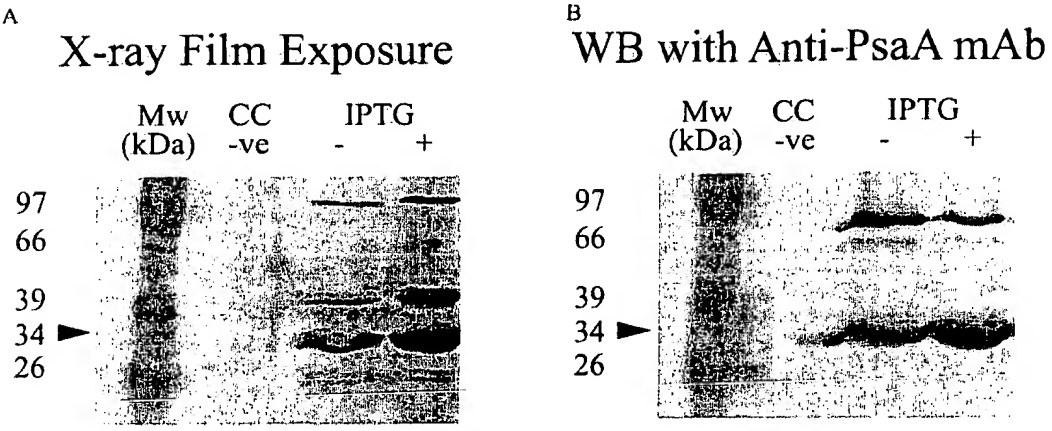

Fig. 4. SDS–PAGE analysis of Triton X^R-114 extracted rPsaA from HOPsaA.7 cells labeled with [^{3}H]-palmitic acid following induction of IPTG. Triton X^R-114-extracted total protein (~10^5 DPM) was electrophoresed on 12% polyacrylamide gels and transblotted to nitrocellulose membranes. Following 10 days' exposure to X-ray film (A) the same membrane was analyzed by Western blot (B) using an anti-PsaA mAb (1E7). Lane MW protein markers; lane CC, normal *E. coli* cell control; lanes – and +, Triton X^R-114 extracts from cells induced without and with IPTG induction, respectively. Arrow indicates [^{3}H] labeled rPsaA.

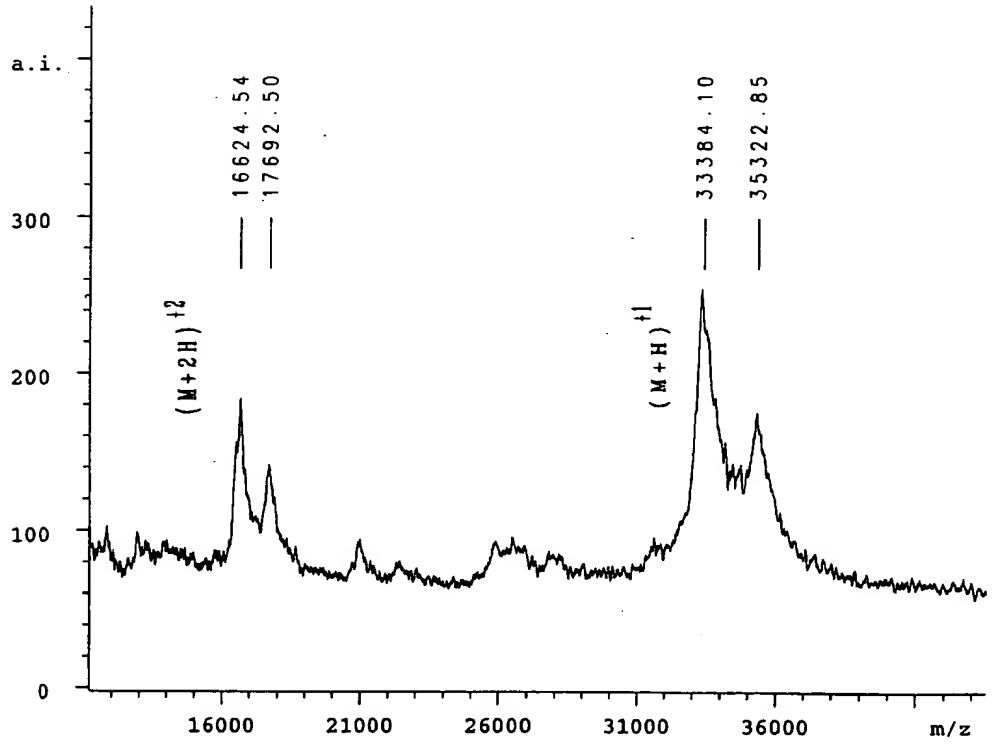

Fig. 5. MALDI-TOF spectrum of rPsaA. The spectrum indicates two major recombinant polypeptides (33,384 Da and 35,322 Da). The estimated mass values (m/z) plotted on the x-axis, and average intensity (a.i.) corresponds to the y-axis.

3.4. Animal data

Mice immunized i.n. at doses of 150 and 500 ng with purified rPsaA (with or with without CTB) were challenged i.n. 6 weeks post-dose 3. Intranasal carriage and bacteremia were determined 1 week post-challenge. We detected both mucosal and systemic specific antibody to rPsaA 2 weeks after dose 3 of rPsaA (data not shown). Significant reduction in carriage ($P < 0.05$, vs controls) was observed in the immunized groups ($P < 0.01$).

Mice that were immunized parenterally with rPsaA (5 μg) demonstrated significant protection against i.n.

carriage ($P < 0.004$) 7 days after challenge (data not shown).

4. Discussion

Bacterial lipoproteins confer many biological functions following infections [31]. Some of them also act as immunogen(s) eliciting protective antibody. For example, a lipidated outer surface protein A (OspA) of *B. burgdorferi* is now considered an effective vaccine against Lyme disease [35,36]. We identified PsaA as a species-common lipoprotein of *S. pneumoniae*, and our preliminary studies suggested its usefulness as an immunogen protecting mice following challenge with virulent pneumococcal strains. However, this lipoprotein is expressed in low quantities in its native form. Therefore, in order to examine the role(s) this lipoprotein may play as a possible third-generation pneumococcal vaccine, we cloned and expressed chimeric *psaA* operon with *B. burgdorferi ospA* signal sequence into protease-deficient *E. coli* cells (HMS174-DE3/pLysS). The identity of recombinant lipoprotein was confirmed by meeting the following criteria: (1) anti-PsaA antibodies recognized rPsaA polypeptide; (2) rPsaA partitioned into Triton X^R-114 detergent phase; (3) [^{3}H]-palmitic acid incorporated into rPsaA polypeptide as shown by Western blot analysis using an anti-PsaA mAb; and (4) mass spectrometry analysis of rPsaA revealed a major peak $(M+H)^{+1}$ of mass 33,384 Da corresponding to palmitoylated rPsaA (theoretical mass of lipidated rPsaA = 33,361 Da).

The native PsaA leader peptide appeared inefficient in producing large quantities of functional PsaA in *E. coli* host [14]. So, in order to achieve greater levels of expression and production of large quantities of functional PsaA, the native leader sequence was replaced with *B. burgdorferi ospA* signal sequence and expressed under the control of T7 promoter. Recently, this OspA signal peptide and also T7 promoter were used to produce large quantities of functional lipidated OspA polypeptide in a genetically modified *E. coli* host [23]. Furthermore, our comparative amino acid sequence revealed OspA peptide as smaller and less hydrophobic than PsaA signal peptide (total amino acids 16 vs 19, and hydrophobic amino acid 10 vs 13).

The role(s) of signal peptidase II depends on the structure of signal peptide, which plays a major role(s) during post-translational modifications and transportation of functional protein in the *E. coli* host [31] . High levels of recombinant OspA expression in *E. coli* might suggest that the host signal peptidase II cleave the OspA signal peptide more efficiently and exports mature recombinant protein to the cellular membrane as related to the native signal peptide, which might be inefficiently processed by the host enzymes. In our study, we selected an *E. coli* host (HMS174-DE3/pLysS) as suitable for expression of rPsaA. This host strain contains a plasmid (pLysS) expressing T7 lysozyme [37] which, in conjunction with host signal peptidase II, facilitates high levels in expression and accumulation of mature PsaA in the cell membrane. Limited expression of T7 lysozyme also enhances cell lysis following detergent treatment. The *E. coli* chimeric construct produced mostly cell-associated recombinant polypeptides (>95%) with an average yield of 10 mg per liter of fermentation culture. Although this is on average a 100-fold higher yield than native PsaA expressed by *S. pneumoniae* as reported earlier [20], our total yield of purified lipidated rPsaA approximately 5 mg/l of fermentation culture.

The initial isolation of rPsaA from the cell pellet was achieved following successive lysozyme treatment, sonication and repeated detergent extraction of rPsaA with 2% Triton X^R-114 buffers, allowing the majority of recombinant proteins (>60%) to partition in Triton X^R-114 phase. The limited solubility of rPsaA in a high concentration of Triton X^R-114 (2%) suggests that the acylated rPsaA formed rigid lipid membrane complexes with the cellular lipoproteins [31]. By detergent phase separation at 37°C followed by extensive dialysis (10 mM phosphate, pH 6.5) most of the host proteins were removed, but there were still detectable amounts of Triton X^R-114, *E. coli* host proteins, and LPS in the rPsaA preparation. The isoelectric point of rPsaA (pI 5.0) helped to optimize the final purification of rPsaA based on basic anionic exchange filter (Q100) chromatography. By washing with 10 mM phosphate buffer (pH 7.6) the majority of impurities, including Triton X^R-114, were removed and the Q100 membrane retained the majority of rPsaA (>90%), which was eluted efficiently with a buffer (100 mM NaCl, 10 mM phosphate, pH 6.5). Because rPsaA is highly hydrophobic and adhesive in nature, we increased the efficiency of rPsaA elution by incorporating Triton X^R-100 (0.1%) and ethanol (20%) in the buffer systems which yielded highly pure (>90%) rPsaA at a concentration of 150 μg/ml. Further concentration of purified rPsaA was achieved by Centricon microfiltration, which increased the concentration of rPsaA 5–10 fold (~1 mg/ml) and also removed salts as well as Triton X^R-100. Our purification protocol appeared to be a convenient, efficient and cost-effective process producing high yields of rPsaA.

Our preliminary data suggest that HOPsaA.7 cells also expressed a slowly migrating rPsaA (probably a dimeric form of rPsaA) polypeptide and a partially processed ~32 kDa along with the monomeric rPsaA. This phenomenon might be explained by incomplete post-translational modifications including acylation, as are often noticed in other prokaryotic expression systems [29]. Recombinant PsaA polypeptides expressed

Table 1
Intranasal immunization with rPsaA and intranasal challenge with the PLN strain of *Streptococcus pneumoniae*[a]

Immunogen	Dose in 10 μl	Number of mice	% dead	% bacteremia or dead	% of live mice with carriage (*P* vs none)[d]	Median log CFU carried[b]	Geometric mean log CFU carried ± S.E.[c] (*P* vs none)[e]
PsaA	500 ng	20	15	20	35.3 (0.036)	< 1	1.44 ± 0.22 (0.024)
PsaA	150 ng	10	10	20	33.3 (0.016)	< 1	1.82 ± 0.46 (0.110)
PsaA	All immune[f]	30	13	20	34.6 (0.009)	< 1	1.75 ± 0.25 (0.018)
None[g]	–	19	21	52	80.0	3.05	2.91 ± 0.31

[a] Mice were immunized on day 0, 7, and 17 with the indicated amount of PsaA. Half of the mice received CTB with the first two injections. Since the results were not dependent on whether or not CTB was used the data with and without CTB have been pooled. All mice were challenged 38 days after the last immunization and sacrificed 7 days later to determine the numbers of CFU in their nasal wash and blood.
[b] < 1 indicates that no CFU were observed in over half the mice.
[c] To calculate geometric means mice with < 1 log CFU recovered from their nose were assigned a value of 0.9.
[d] Statistical differences were calculated by a two-tailed Fisher exact test.
[e] Statistical differences were calculated by a two-tailed Wilcoxin two-sample rank test.
[f] Includes all mice immunized with 500 ng and those immunized with 150 ng.
[g] The carriage CFU of one control mouse grew a contaminant, thus obscuring the possibility of detecting pneumococci. Only the data from the 19 remaining mice were included in the analysis.

by either prokaryotic or eukaryotic systems, overall, differ in molecular mass [14], but remain similar in immunogenecity to the native PsaA by Western blot analysis (Fig. 1 and Fig. 3).

Interestingly, rPsaA polypeptide (34 kDa) was hydrophobic, lipidated, and aggregated protein complexes (micelle) closely resembling the biological and biochemical functions of other bacterial lipoproteins [29–34] in several ways. First, [^{3}H]-palmitic acid incorporated into rPsaA following post-translational modifications by *E. coli* cells, and the labeled rPsaA partitioned into the Triton X^R-114 phase. Second, the inability to analyze N-terminal sequences by the Edman technique of both native and rPsaA polypeptides indicated an N-terminus block. The majority of gram-positive bacteria, including pneumococcal strains, express very low levels of membrane bound acylated proteins which contain a lipoamino acid, N-acyldiacylglycerylcysteine, at the N terminus following post-translational modifications [25]. These bacterial lipoproteins contain two or three molecules of palmitic acid (Pam_3Cys, 60%) along with other fatty acid moieties attached to a N terminus cysteine residue [29–34]. Furthermore, the computer-deduced amino acid sequence of PsaA signal peptide of several *S. pneumoniae* strains [28] revealed the presence of a consensus sequence (-Leu-Val-Arg-Cys-), which closely resembles the sequence (-L-X-Y-C-) delineated by Jenkinson and Lamont to be a site of lipidation as well as a cleavage site by signal peptide II for streptococcal lipoprotein receptor antigen I proteins [33]. A comparative amino acid analysis also revealed that OspA has a similar hydrophobic consensus sequence (-L-I-A-C-), but it has 10 hydrophobic amino acids as compared to that of 13 for the PsaA signal peptide. This might explain the low levels of expression of PsaA by the pneumococcal strains as an indication of the inability of its signal peptide to express large quantities of recombinant PsaA polypeptides in the *E. coli* vector system [14]. It is also possible that the palmitoylation is not as efficient a process in *S. pneumoniae* as compared with that in a protease deficient *E. coli* host system.

In general, the acylated bacterial proteins are immunogenic and participate in activation of B-cells and T-cells, eliciting protective antibodies along with other biological functions [38–40]. Our initial studies indicated that lipidated rPsaA was immunogenic, similar to native PsaA isolated from *S. pneumoniae*. We observed antibody production both in peripheral blood or mucosal secretions of mice given purified rPsaA intranasally (Table 1). We also noticed significant protection (reduction in carriage) against the lethal challenge of *S. pneumoniae* in animals following immunization with rPsaA (Table 1). Further studies to determine the dose and range of protection are on-

going. Our preliminary data suggest that rPsaA polypeptide acts effectively in protecting mice.

We have developed a simple prokaryotic expression system which provides a means of generating a high-quality purified rPsaA polypeptide (~5 mg/l of fermentation culture). Additionally, our data strongly indicate that rPsaA is palmitoylated, immunogenic and closely resembles native PsaA. The ability to produce purified rPsaA in these quantities will enable evaluation of this protein as a potential third-generation vaccine candidate alone or in combination with other pneumococcal antigens. Further knowledge of the structure and function of rPsaA will enable an appropriate design of studies to optimize purification schemes and investigate the relationship of structure to immunogenecity and will ultimately aid in the prevention of pneumococcal disease.

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